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STRATIFICATION IS NECESSARY FOR SUCCESSFUL CRYOPRESERVATION OF AXES FROM STORED HAZELNUT SEED

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SUMMARY

Stratification of stored hazelnut seed improved shoot production from both control and cryopreserved isolated embryonic axes. Axes from freshly harvested nuts grown *in vitro* had viabilities of 90% to 100% with shoot production of 60% to 80% but shoot production decreased to near 30% for both control and cryopreserved axes from seed stored for one month. Shoot growth of axes from seed stored for four months increased from 33% to 94% when seeds were stratified for two weeks before axis removal. Axes from stratified-stored seed were dried to 8% moisture under laminar flow and cryopreserved with resulting 85% viability and 70% shoot growth rates, while only 30% of unstratified axes produced shoots.

KEY WORDS: stratification, embryonic axes, germplasm, hazelnut, *Corylus*, cryopreservation

INTRODUCTION

Hazelnuts (*Corylus spp.*) are an important temperate nut crop with seeds that are considered desiccation-tolerant LN2-sensitive in their storage behavior (1). Development of new cultivars resistant to insects or diseases or with improved nut characteristics relies on breeding material from wild species (2). Wild hazelnut germplasm must be preserved as growing plants in orchards or as refrigerated or frozen pollen due to the short viability of the seeds. Hazelnut seeds remain viable for six months to one year in storage (2). In addition to their large size, hazelnut seeds have a hard seed coat, internal dormancy and irregular germination with increasing lengths of storage (3). Induction of embryo dormancy occurs within 24 days of harvest when hazelnut seeds are stored under dry

conditions and is due to the movement of inhibitors from the testa and pericarp into the axis (4). Removal of the dormancy requires chilling moist seed or applying gibberellic acid (GA_3) (4).

Storage of *Corylus* germplasm in liquid nitrogen would provide a long term base collection. *Corylus* seeds do not survive temperatures below -40°C (1). A recent report indicates that embryonic axes of freshly harvested hazelnuts can be successfully stored in liquid nitrogen with good regrowth potential (6). Embryonic axes of *Hevea* and *Coffea* survived freezing only when the water content was below 20% (5, 7). *Quercus faginea* embryonic axes at 20% water content had high survival rates (8), while embryonic axes of mature stored *Aesculus, Castanea, Corylus* and *Quercus* seeds remained viable following cryopreservation but varied in their growth responses (9). Initial tests with *Corylus* seed showed that isolated embryonic axes of stored seed remained viable after cryopreservation, but shoot production declined compared to fresh seed (Reed and Normah, unpublished). This study was designed to determine procedures necessary for successful cryopreservation and regrowth of embryonic axes of stored seed of *Corylus avellana* L. cv. Barcelona with the goal of application to germplasm storage.

MATERIALS AND METHODS

'Barcelona' hazelnuts were obtained at the beginning of harvest season on Sept. 18, 1993 from Wayne Chambers, Albany, Oregon. The nuts were fully mature and collected from the ground. This cultivar was chosen because it was readily available in large quantities. Nuts were sorted from any foreign materials, then packed into 70 liter burlap bags. The bags were stored at 20°C and ambient relative humidity (20-40%). Moisture content of axes was determined on a fresh weight basis. Embryonic axes were weighed, dried in an oven at 104°C for 16 hr and reweighed.

Seed germination. The nuts were mechanically cracked to remove some or all of the pericarp and soaked in a solution of GA_3 (10 mg/liter) for 24 hr. The germination test was performed using the between paper method (10) in a seed germinator with alternating temperatures of 10°C (14 hr.)/25°C (10 hr.) under a 12 hr. photoperiod. The seeds were considered viable when radicles emerged. Percentage radicle emergence was determined after four weeks. Seeds without radicle emergence but which were not moldy were tested for viability using the tetrazolium test (2,3,5-triphenyltetrazolium chloride, 1.0%) (10).

Determination of growth regulators for Corylus embryonic axes. The axes were excised, surface sterilized in 10% bleach (final concentration 0.53% sodium hypochlorite) for 10 min, rinsed in sterile water, and placed in individual wells of 24-well plates on NCGR-COR medium (11) with 3% glucose, 0.6% agar (Difco Bitek Agar, Detroit), with or without N^6-benzyladenine (BA), indole-3-butyric acid (IBA), and gibberellic acid (GA_3) (Sigma Chemical, St. Louis, MO). Axes were considered viable when they enlarged and turned green. Growth room conditions were 25°C with a 16 hr photoperiod (25μmol$\cdot m^{-2}\cdot s^{-1}$).

Growth regulator tests. 1. Sept. 25, 1993. a) no growth regulators b) BA 1.5 mg/l + IBA 0.01 mg/l c) BA 5 mg/l. Two replicates with 24 axes for each treatment per replication were used. 2. Sept. 30, 1993. Medium with BA 1.5 mg/l + IBA 0.01 mg/l a) no GA_3 b) GA_3 0.1 mg/l c) GA_3 0.5 mg/l. Three replicates with 20 axes each were used. 3. Oct. 22, 1993. a) no growth regulators b) BA 1.5 mg/l + IBA 0.01 mg/l + GA_3 0.1 mg/l c) GA_3 0.1 mg/l d) Soaking the axes for 5 min. in a solution of 10 mg GA_3 /100 ml H_2O then planting on medium with no growth regulators. Three replicates with 20 axes each were used. 4. Nov. 12, 1993. a) no growth regulators b) BA 0.1 mg/l c) BA 0.5 mg/l d) BA 1.0 mg/l e) BA 1.5 mg/l. For each treatment three replicates of 10 axes each were soaked in GA_3 at 10 mg/100ml water for 5 min and three replicates were cultured directly.

Desiccation under laminar flow and cryopreservation. Nuts were mechanically cracked and the pericarp was removed. The embryonic axes were excised, surface sterilized, blotted dry on sterile filter paper and dried in an open Petri dish in a laminar flow hood for 0, 1, 2, 3, and 4 hr. After each desiccation hr., three replicates of 10 axes (30 total per drying time) each were placed into cryovials, immersed directly into liquid nitrogen and stored for one hr. Cryovials were thawed in a water bath at 45°C for one min. and axes cultured on NCGR-COR medium. The moisture content was also determined.

Desiccation of axes over silica gel. The axes were excised and sterilized as above. After sterilization, the axes were placed in sealed desiccators over 125 g silica gel for 0, 1, 2, 3, and 4 hr., cryopreserved, thawed, and cultured as in the laminar flow experiment. The moisture content was also determined.

Chilling treatment of isolated embryonic axes from four-month stored seed. NCGR-COR medium with no growth regulators was tested with five lengths of chilling in the refrigerator at 4°C (0, 2, 4, 6, and 8 weeks). Three replications of 20 axes each were used. Axes were dissected and surface sterilized as stated earlier, placed on medium in 24-well plates and stored in the refrigerator for the stated time periods. At the end of the cold treatment the plates with the axes were transferred to the growth room and grown for 4 weeks before data were taken.

Stratification tests of four-month stored seed. Seeds were surface sterilized in 20% bleach for 10 minutes and rinsed in tap water. Perlite was moistened with tap water, autoclaved for 30 min and cooled. Intact seeds were planted in the perlite and the tray enclosed in a plastic bag, placed in the cold room and chilled at 4°C for 0, 2, 4, 6 and 8 wk. before removal and culture of the embryonic axis. At each interval the seeds were removed, manually cracked and either placed in the germinator (with alternating temperatures of 10°C (14 hr)/25°C (10 hr) and 12 hr. photoperiod) or the axes were dissected, surface sterilized and cultured on medium without growth regulators. Twenty seeds or axes per treatment were used for each of 3 replicates.

Cryopreservation of stratified axes from four-month stored seed. Another set of intact seeds were chilled in perlite as above for use with cryopreservation tests. After two weeks of chilling treatment, nuts were manually cracked and the embryonic axes excised and surface sterilized. The axes were blotted dry and dried under laminar flow for 0, 1, 1.5, and 2 hr. After each desiccation period, three replicates of 10 axes each were placed in cryovials, frozen and thawed as above. Axes were recorded as viable when they expanded and turned green and as growing when a normal shoot was produced. A set of 30 axes was cultured without cryopreservation treatment as controls and moisture determination was carried out on 30 additional axes.

RESULTS AND DISCUSSION

Axis growth in culture. Control axis viability remained high for the duration of the study but shoot production decreased and callus formation increased in axes cultured during the first month of storage (Figure 1). These results are similar to those seen by Pence (9) who found that axes from stored seed remained viable after cryopreservation, but had little or no shoot regrowth.

Shoot production from axes of two-month stored seed increased with increasing BA concentrations (Figure 2). BA at 0.5 to 1.5 mg/l is commonly used in hazelnut micropropagation (11) and significantly more shoots were produced by axes on medium in that range of concentrations than those on medium without BA. Gonzalez-Benito and Perez (6) attributed callus production on recovering axes to high growth regulator concentrations in the medium. For 'Barcelona' axes, callus is more likely due to storage or dormancy. Axes from stored seeds grown on medium with no growth regulators callused (turned green, swelled and sometimes produced surface callus tissue) at the same rate as axes on medium with growth regulators (data not shown). The addition of IBA to the media did not affect shoot growth or callus production in the axes.

Jarvis (12) found microdrops of GA_3 stimulated growth of isolated axes with one cotyledon attached but in our tests GA_3, either in the medium or as a soak, was ineffective in increasing shoot growth of isolated axes without a cotyledon attached (data not shown). GA_3 is used to overcome the inhibition of germination in hazelnut seed (13) and was successful in promoting germination in whole 'Barcelona' seeds within the same storage group (data not shown). The response of hazelnuts to gibberellins in breaking dormancy involves elongation of the cotyledonary petiole (14). Jarvis and Shannon (15) found GA_3 to stimulate synthesis of poly(A) RNA in the embryonic axis and speculated that this might influence the breaking of dormancy, but in our experiments GA_3 treatment did not influence shoot production of the embryonic axes.

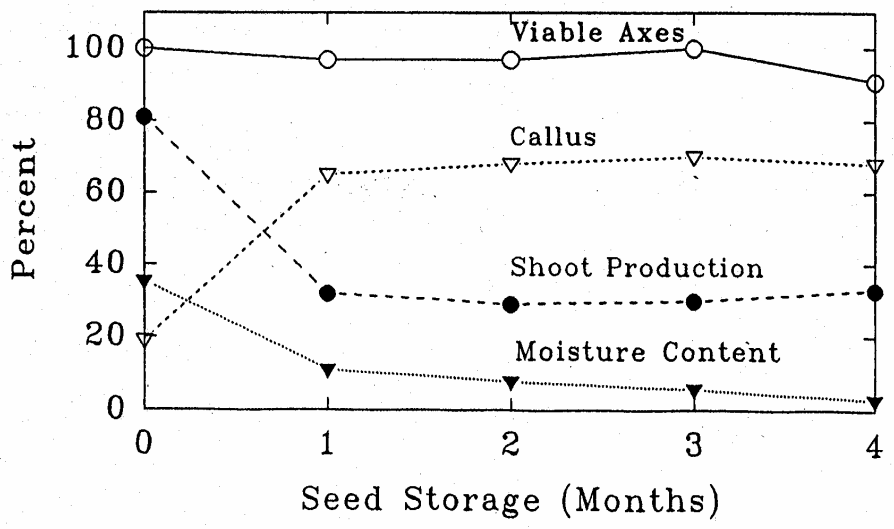

Figure 1. Viability, shoot production, callus production and moisture content of embryonic axes of 'Barcelona' hazelnut seed cultured after increasing amounts of storage.

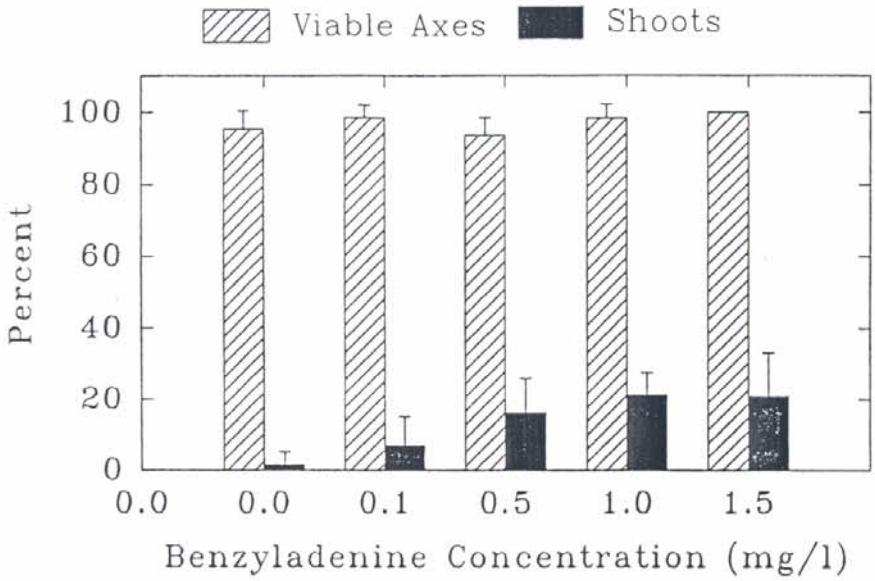

Figure 2. Effect of benzyladenine concentration on viability and shoot production of isolated embryonic axes of 'Barcelona' hazelnut seed stored for 2 months.

Primary dormancy in hazelnut is released by removing the pericarp and testa from the embryo (3). Secondary dormancy, due to inhibitory substances in the testa and pericarp which diffuse into the axis, occurs within 24 days of harvest and requires stratification or GA_3 treatment for seed growth (3). This secondary dormancy was apparent in ' Barcelona' seeds within 2 weeks of harvest and required eight weeks of stratification for high rates of radicle emergence from whole seeds (Table 1).

Table 1. Effects of stratification of four-month stored whole seed or cold treatment of isolated embryonic axes of stored hazelnuts on shoot production of the isolated axes *in vitro*.

Weeks of stratification or cold treatment	% Shoot growth		% Radicle emergence
	Cold-treated axes	Axes from stratified seed	Stratified seed
0	32.8 ± 4.8	32.8 ± 4.8	17.3 ± 4.1
2	18.2 ± 7.9	94.3 ± 2.3	22.7 ± 10.0
4	10.4 ± 8.3	85.0 ± 5.0	18.3 ± 2.9
6	9.1 ± 15.8	86.4 ± 7.7	48.4 ± 12.4
8	5.0 ± 5.0	84.3 ± 4.8	86.7 ± 10.4

Mean ± standard deviation, n = 60

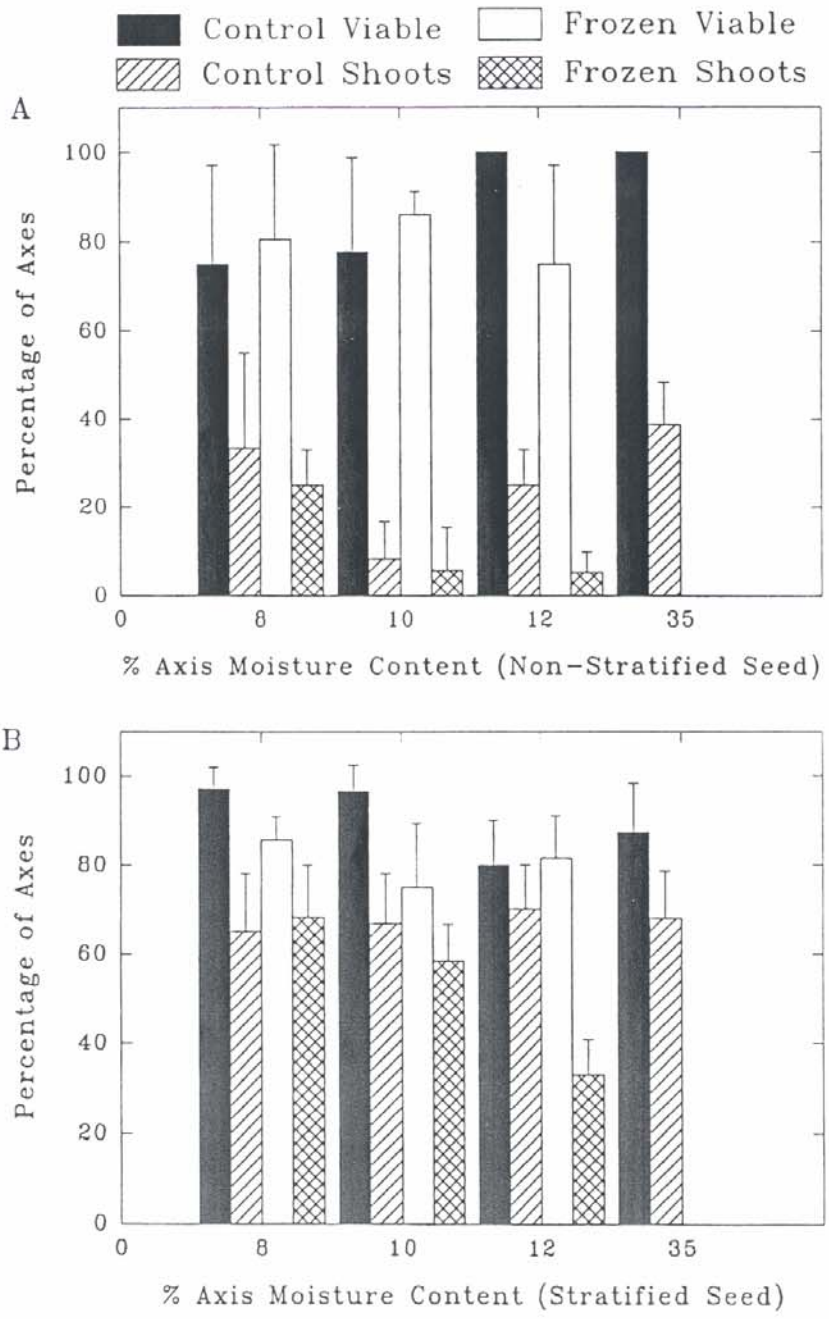

Figure 3. Viability and shoot production from embryonic axes of 'Barcelona' hazelnut seed stored for four months, (A) without and (B) with stratification treatment. Axes were cryopreserved following dehydration to various moisture contents under laminar flow. Stratification treatments of intact seed were for two weeks at 4°C. (n = 60)

Stratification of intact seeds for two or more weeks improved shoot production of cultured-isolated axes while cold treatment on growth medium after the axes were isolated was not successful (Table 1). Jarvis and Wilson (16) found that axes from stratified dormant seeds of 'Kent Cob' hazelnut grew normally only if one-third of a cotyledon was attached, but in our experiments the axes of stratified seed of 'Barcelona' developed normal shoots and roots without cotyledon tissue. Shoot production declined with increased treatment time in isolated axes cold treated in the refrigerator, probably due to dehydration during treatment (data not shown). A preliminary imbibition of the axes before cold treatment might be required for successful growth from refrigerated axes.

Cryopreservation treatments.

Cryopreserved axes from nuts stored four months after harvest and dried to 8-12% moisture under laminar flow had high viability and low shoot formation similar to the non-frozen controls (Figure 3A). Axes cryopreserved without dehydration (35% moisture content) did not survive. Axes from two month stored seed dried in laminar flow or over silica gel to moisture contents of 8% to 13% before cryopreservation gave results similar to the four month data (data not shown). Preliminary tests with alginate-bead dehydration (17) and propanediol (18) methods had no surviving axes following cryopreservation and produced low survival rates in the control axes.

Axes from seed stratified for 2 weeks following four months of storage had high rates of viability and shoot production when dehydrated to 8% or 10% moisture and cryopreserved (Figure 3B). Shoots were produced by over 60% of axes of both control and frozen treatments dried to 8-10% moisture. Survival of cryopreserved axes at 12% moisture was significantly lower than at 8-10% and there was no survival at 35% moisture content. A recent study of laminar flow drying and cryopreservation of fresh axes of *C. avellana* L. cvs. Butler and Morell indicated the highest shoot production for axes with moisture contents below 15% (6).

CONCLUSIONS

Culture and cryopreservation of hazelnut embryonic axes is complicated by decreased shoot production in axes from seed stored for even a short time. A two week stratification of stored seeds before excision of axes greatly improved shoot growth and decreased callusing by cultured axes. Drying isolated embryonic axes to 8-10% moisture, either by laminar flow or over silica gel, produces high survival and shoot growth rates from stratified-stored *Corylus* seed. Initial tests with growth of axes from stored seed showed that shoot production improved when benzyladenine was added to the medium. This should be tested on axes from stratified seed to further improve shoot production following cryopreservation.

Whole hazelnut seeds do not survive immersion in liquid nitrogen (1), so excision and storage of the axis is a good alternative. If fresh axes are not readily available, short stratification treatment of stored nuts is a relatively simple, alternative technique for long-term storage of hazelnut germplasm.

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